

04/95, 381
08/04, 068

APPLICATION FOR UNITED STATES PATENT



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Invention: 50 AN ADHESION MOLECULE

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METHOD OF INHIBITING HIV INFECTION
WITH CD44 AND ANTI-CD44 ANTIBODIES

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SPECIFICATION

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METHOD OF INHIBITING
HIV INFECTION WITH
ANTI-CD44
ANTIBODIES

This is a continuation-in-part of Application CD44 AND
No. 07/669,730, filed March 15, 1991.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to methods of suppressing T cell activation, inhibiting CD44-mediated cell adhesion and CD44-monocyte IL1 release, treating inflammation, and transporting a drug or cytotoxic agent to a site of inflammation.

10 Background Information

Recent work has defined the importance of cell adhesion molecules in immune cell function (reviewed in Springer (1990) Nature 346:425-434; Haynes et al. (1989) Springer Sem. Immunopathol. 11:163-185; Hemler (1988) Immunol. Today 109-113). Cell adhesion molecules have been described that are receptors for soluble molecules (Haynes et al (1984) Nature 312:763-766), receptors for viruses (reviewed in Springer (1990) Nature 346:425-434; Dalglish et al. (1984) Nature 312:763-766; Klatzmann et al. (1984) Science 225:59-63), and ligands for other cell surface molecules (reviewed in Springer (1990) Nature 346:425-434; Haynes et al. (1989) Springer Sem. Immunopathol. 11:163-185; Hemler (1988) Immunol. Today 109-113).

On immune cells, cell adhesion molecules mediate a wide variety of normal cell functions including cell movement, adherence to other cells, adherence to extracellular matrix proteins, mononuclear cell homing and monocyte cytokine release (reviewed in Springer (1990) Nature 346:425-434; Haynes et al. (1989) Springer Sem.

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Immunopathol. 11:163-185; Hemler (1988) Immunol.
Today 109-113; Haynes et al. (1989) Immuno. Today
10:423-428). The CD44 molecule has been of recent
interest because this protein has multiple
5 proinflammatory functions, exists in soluble form
in serum and plasma, and regulates the function of
other adhesion molecules (reviewed in Haynes et
al. (1989) Springer Sem. Immunopathol. 11:163-
185; Haynes et al. (1989) Immuno. Today 10:423-
10 428).

The CD44 molecule is an 85kd glycosylated
molecule with N-terminal sequence homology to
cartilage link proteins (Stamenkovic et al (1989)
Cell 56:1057-1062; Goldstein et al (1989) Cell
15 56:1063-1072). Forms of CD44 of varying sizes
have been described on many cell types (Haynes et
al (1989) Immunol. Today 10:423-428, Stamenkovic
et al (1989) Cell 56:1057-1062; Goldstein et al
(1989) Cell 56:1063-1072; Jalkanen et al (1988) J.
20 Immunol. 141:1615-1623). Variations in the size
of CD44 isoforms have been suggested to be due to
glycosylation differences, the addition of
chondroitin sulfate molecules to CD44 (Jalkanen et
al (1988) J. Immunol. 141:1615-1623), and in some
25 cases, to alternative splicing of CD44 mRNA
(Dougherty et al (1988) Exp. Hemat. 18:703, St.
John et al (1989) Req. Immunol. 300-310). Three
forms of CD44 have been identified on peripheral
blood mononuclear cells (PBMC) (Hale et al) and an
30 85kd form (presumably a secreted form) has been
identified in serum, plasma (Telen et al (1983) J.
Clin. Invest. 71:1878-1886; Lucas et al (1989)
Blood 73:596-600) and now synovial fluid.

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Functionally, the CD44 molecule has been shown
35 to be a central molecule involved in T lymphocyte
adhesion, T lymphocyte activation and monocyte
cytokine release (Haynes et al (1989) Immunol.

Today 10:423-428; Jalkanen et al (1986) Science
 233:556-558; Jalkanen et al (1987) J. Cell Biol.
 105: 983-990; Aruffo et al (1990) Cell 61: 1303-1313;
 Miyake et al (1990) J. Exp. Med. 172:69-75; Lesley
 5 et al (1990) Exp. Cell Res. 187:224-233;
 Stamenkovic et al (1989) Cell 56:1057-1062;
 Goldstein et al (1989) Cell 56:1063-1072; Jalkanen
 et al (1988) J. Immunol. 141:1615-1623). The
 association of the CD44 intracellular domain with
 10 the cytoskeletal protein, ankyrin, and with the
 enzyme protein kinase C (PKC) (Kalomiris et al
 (1989) J. Biol. Chem. 264:8113-8119) has suggested
 a role for CD44 in signal transduction of surface
 events to intracellular molecules. Ligand binding
 15 to the CD44 molecule promotes T cell adherence to
 monocytes via other adhesion molecule pathways
 (ICAM-1/LFA-1 and LFA-3/CD2) (Denning et al;
 Koopman et al (1990) J. Immunol. 145:3589-3593)
 suggesting that CD44 can serve as a regulator of
 20 function of other adhesion molecules (reviewed in
 Haynes et al (1989) Springer Sem. Immunopathol.
 11:163-185; Haynes et al. (1989) Immunol. Today
 10:423-8
 109-115).

Recent studies have demonstrated that the CD44
 25 protein is the primary receptor for hyaluronate in
 rodents and humans (Aruffo et al (1990) Cell 61: 1303-
 1313; Miyake et al (1990) J. Exp. Med. 172:69-75;
 Lesley et al (1990) Exp. Cell Res. 187:224-233).
 Both hyaluronate (Hiro et al (1986) Biochem.
 30 Biophys. Res. Comm. 140: 715-722) and CD44 mAB (Denning
 et al (1989) J. Immunol. 142: 2988-97
 et al; Webb et al Science, 249:1295) binding to
 monocytes induces monocyte IL1 release. On T
 cells, hyaluronate and CD44 mAB ligation of CD44
 have disparate effects; CD44 mABs augment T cell
 35 triggering (Denning et al (1989) J. Immunol. 143: 798-801; Shimizu et al (1989) J. Immunol.
 143:2457-2463) while hyaluronate suppresses T cell

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triggering (Anastassiades et al (1984) Rheumatol.
11:734-729). Finally, CD44 mabs and polyclonal
anti-CD44 serum have been shown to inhibit the
binding of lymphocytes to high endothelial venules
5 in inflammatory sites such as synovium (Jalkanen
et al (1986) Science 233:556-558; Jalkanen et al
(1987) J. Cell Biol. 98¹⁰⁵:983-990; Jalkanen et al
(1988) J. Immunol. 141:1615-1623), suggesting
lymphocyte CD44 is one of several molecules
10 involved in organ-specific lymphocyte homing.
Thus, the hyaluronate receptor (CD44) molecule, by
existing in several isoforms, and by virtue of
wide cellular distribution, functional association
with other adhesion molecules, and physical
15 association with ankryin and PKC, is a
multifunctional proinflammatory molecule involved
in immune cell activation (reviewed in Haynes et
al (1989) Immunol. Today 10:423-428).

Hyaluronate, the ligand for CD44, is an
20 important component of synovial fluid and plays a
critical role in maintaining high viscosity of
synovial fluid in normal diarthroidal joints
(reviewed in Schuber and Hammerman (1964) Bull.
Rheum. Dis. 14:345-348; Castor et al (1966) Arth.
25 Rheum. 9:783-794). In rheumatoid arthritis (RA),
synovial fluid, hyaluronate concentration and
degree of polymerization is decreased (Castor et
al (1966) Arth. Rheum. 9:783-794). Reduction in
synovial fluid hyaluronate concentration and
30 degree of polymerization has been suggested to be
an important factor leading to joint dysfunction
and destruction in RA (Schubert et al (1964) Bull.
Rheum. Dis. 14:345-348; Castor et al (1966) Arth.
Rheum. 9:783-794), and potentially may decrease
35 the immunosuppressive effect of hyaluronate on T
cells (Anastassiades et al (1984) Rheumatol.
11:734-729).

Applicants have demonstrated that CD44 is upregulated in RA on many synovial cell types and that the level of CD44 present in synovial tissue is directly proportional to the degree of synovial inflammation in RA. Applicants have also demonstrated that CD44 is immunosuppressive to T cells. The present invention relates, at least in part, to a method of interdiction of proinflammatory functions of the CD44 molecule.

SUMMARY OF THE INVENTION

It is a general object of this invention to provide a method of treating inflammation.

It is a specific object of this invention to provide a method of suppressing T cell activation.

It is another object of the invention to provide a method of inhibiting CD44-mediated cell adhesion or CD44-mediated monocyte IL1 release.

It is yet another object of the invention to provide a method of transporting a drug or cytotoxic agent to a site of inflammation, and to compositions suitable for use in such a method.

Further objects and advantages of the present invention will be clear from the description that follows.

In one embodiment, the present invention relates to a method of suppressing T cell activation in an human comprising administering to the human CD44 protein peptide or derivative thereof in an amount sufficient to suppress T cell activation.

In another embodiment, the present invention relates to a method of inhibiting CD44-mediated cell adhesion or CD44-mediated monocyte IL1 release in an animal comprising administering to

the human CD44 protein or peptide or derivative thereof in an amount sufficient to inhibit CD44-mediated cell adhesion or CD44-monocyte IL1 release.

5 In a further embodiment, the present invention relates to a method of treating inflammation in an human comprising administering to the human CD44 protein or peptide or derivative thereof in an amount sufficient to reduce the inflammation.

10 In another embodiment, the present invention relates to a method of transporting a drug or cytotoxic agent to a site of inflammation in an human comprising administering to the human CD44 protein or peptide or derivative thereof linked to
15 the drug or cytotoxic agent. In a preferred embodiment, the CD44 protein or peptide or derivative thereof and the drug or cytotoxic agent are incorporated into a liposome.

BRIEF DESCRIPTION OF THE DRAWINGS

20 Figure 1. Upregulation of CD44 Expression in RA But Not in Non-inflammatory OA or Trauma. Figures 1A, B, and C are from trauma synovium no. 229 (inflammation score = 5). Panel A shows
25 hematoxylin and eosin (H and E) stain, panel B shows reactivity of synovium with anti-fibronectin mAb FN15 (fibronectin index = 2+) and panel C shows reactivity of synovium with anti-CD44 mAb AlG3 (CD44 index = 1+). Figures 1D, E, and F are
30 from OA synovium no. 36 (inflammation score = 5). Panel D shows H and E stain, panel E shows fibronectin expression (fibronectin index = 1+), and panel F shows CD44 expression (CD44 index = 1+). Figures G, H, and I are from RA no. 86
(inflammation score = 18). Panel G shows H and E
35 stain, panel H shows fibronectin expression

(fibronectin index = 4+) and panel I shows CD44 expression (CD44 index = 3+). Figures J, K, and L show RA synovium no. 7 (inflammation score = 13) with pannus formation. Figure J shows H and E stain, Figure K shows fibronectin expression (fibronectin index = 4+) and Figure L shows CD44 expression (CD44 index = 4+). All panels showing fibronectin and CD44 expression are indirect IF. All panels 400X.

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10 Figure 2^{A-B} Quantitative Western Blot Analysis
of CD44 Protein in Synovial Tissue. Equal amounts
of tissue were extracted from each synovium and
run on SDS-PAGE followed by Western blot analysis
with anti-CD44 mab A3D8. Figure 2A shows Western
15 blot of OA synovium no. 198 (inflammation score =
3), trauma synovium no. 229 (inflammation score =
5), and RA
synovium nos. 7, 154, and 86 (inflammation scores
13, 21, and 18, respectively). Figure 2B shows
20 the area under the densitometry curve (arbitrary
units) of the CD44 bands shown in Figure 2A.

Figure 3. Western Blot Analysis of CD44
Protein in Trauma, OA and RA Synovial Fluid.
Lanes A, C, and E are control lanes in which CD44
25 protein was immunoprecipitated with CD44 mab and
then run in Western blot analysis and blotted with
control P3X63/Ag8 IgG1 paraprotein. Lanes B, D,
and F are CD44 protein immunoprecipitated with
CD44 mab and then blotted with CD44 mab. Lanes A
30 and B are from synovial fluid no. 11 (trauma, cell
count 450, relative CD44 level = 1.0) lanes C and
D are from synovial fluid no. 29 (OA, cell count
3,469, CD44 level = 0.77) and lanes E and F from
RA synovial fluid 13 (cell count 11,061, CD44
35 level 1.69).

to effect suppression. Examples of CD44 peptides suitable for use in the present method include those sets forth in Table 1.

Table 1

5 Examples of CD44 Peptides That Can Be Used To Inhibit CD44-Mediated Immune Cell Functions

Peptide no.	Sequence	aa
10 X CD44-1	(C)EKNGRYSISRTEAADCKAFN	(CD44 NO:1) 37-57
X CD44-2	(C)NTSQYDTYCFNASAPPEEDCTS	(CD44 NO:2) 110-131
X CD44-3	(C)RDGTRYVQKGEYRNPEDIYPSNPTDDDVSS	(CD44 NO:3) 150-180
X CD44-4	(C)RDGTRYVQKGEYRNPEDIYPSNPTDDDVSSGSSSERSSTS	(CD44 NO:4) 150-190
X CD44-5	(C)YRTNPEDIYPSNPTDDDVSS	(CD44 NO:5) 161-180
X CD44-6	(C)TVHIPDEDSPIWTDSTPRI	(CD44 NO:6) 200-219
15 X CD44-6a	DSPWITDSTRIFATRDQDTI	(CD44 NO:7) 208-227
X CD44-7	(C)ATRDQDTFHPSGGSHHTHESDGHSHGSQEGGAN	(CD44 NO:8) 221-255
X CD44-8	(C)RDGIRYVQKGEY-PSNPTDD-TSGGYIFYTF	(CD44 NO:9) 150-161 (CD44 NO:10) 170-187 (CD44 NO:11) 189-198
20 X CD44-9	LCLVPLSLAQIDLNITCRFAGVFHVEKNGRY	(CD44 NO:12) 112-42
X CD44-10	LCKAFNSTLPTMAQMEKALSIGFETCRY	(CD44 NO:13) 78-108
X CD44-11	CRYGFIEGHVVIPRIHPNSIC	(CD44 NO:14) 77-97
X CD44-12	RYGFIEGHVVIPRIHPNSI	(CD44 NO:15) 76-96
X CD44-13	LTYNTSQYDTY	(CD44 NO:16) 107-117
25 X Sequences from Stamenkovic et al, <u>Cell</u> 56:1057-1062, 1989.		

Administration can be by injection or topical application (for example topically applied to the eye). Injection can be made directly into a skin lesion.

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Figure 4. Comparison of RA Synovial Fluid Cell Counts Versus Relative Levels of Synovial Fluid CD44 Protein.

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Figure 5^{A-B}. Western Blot Analysis of CD44 Protein in Synovial Fluid from non-RA Types of Inflammatory Synovitis. Figure 5A compares CD44 levels in gout versus trauma synovial fluid. Control lanes A and B as in Figure 3. Lane B shows CD44 in fluid 11 (CD44 level = 1.0) and lane D shows CD44 in fluid 32 (CD44 level = 3.79). Band at 40 kd in lanes A, B, and C is a non-specific band not present in lane D for technical reasons. Figure 5B compares CD44 levels in psoriatic arthritis versus trauma synovial fluid. Control lanes A and C as in Figure 3. Lane B is trauma fluid no. 11 (CD44 level = 1.0) lane D is psoriatic arthritis synovial fluid no.100 (CD44 level = 7.0).

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Figure 6. Shows a western blot analysis of recombinantly produced CD44-Rg-2 protein that could be used as an immunosuppressive agent to inhibit CD44-mediated proinflammatory functions.

DETAILED DESCRIPTION OF THE INVENTION

25
The present invention relates to a method of treating inflammation and immune-mediated tissue damage, such as occurs, for example, in the course of autoimmune diseases.

30
In one embodiment, the present invention relates to a method of suppressing T cell activation in an human comprising administering to the human the CD44 protein, or derivative or peptide portion thereof, in an amount sufficient

An additional form of the CD44 molecule that may be used as an immunosuppressive agent is a recombinantly produced CD44 molecule or a portion of the CD44 molecule produced by recombinant DNA technology. An example of such a form of CD44 has been reported by Aruffo, A et al Cell 61:1303-1313, 1990. This form of CD44 has been recombinately engineered to contain portions of the immunoglobulin protein constant domains. The addition of immunoglobulin domains to the extracellular domain of CD44 yielded in molecule called CD44-Rg-2 that has the properties of being seclused by COS cells when a plasmid containing this CD44-Rg2 gene was transfected into COS cells (Aruffo et al. Cell 61:1303-1313, 1990). The presence of immunoglobulin on the extracellular domain of CD44 would also have the potential advantage of increasing the circulating half-life of the CD44 molecule when administered to humans or animals.

Production of CD44-Rg-2 fusion construct: CD44-Rg-2 plasmid can be transfected into COS cells using DEAE dextran as described in Seed and PNAS 84: 3365-3369, 1987 and Aruffo, A et al Cell 61: 1303-1313, 1990. Semi-confluent COS cells plated on 100mm plates will be transfected. Twelve hours after transfection, cells are trypsinized, seeded onto fresh 100mm dishes and allowed to grow for 7-10 days. On the fourth day 5ml fresh media, 10% calf serum are added per dish. Supernatants are harvested and stored at 4°C .

Purification of CD44-Rg protein: Twelve hours following transfection, a fraction of the COS cells transfected are seeded into flasks. Thirty-six hours post-transfection, the cells are washed with PBS and overlaid with ~~cysteine~~ ^{Cysteine}-methionine

media for 30 min. [³⁵Methionine and [³⁵S]Methionine and [³⁵S] Cysteine will be added to a final concentration of 150 µCi/ml, and the cells will be allowed to incorporate the label overnight. The supernatants will be harvested and incubated with 200 µl of protein A-Trisacryl at 4°C for 12 hours. The beads will be collected by centrifugation and washed in 10 ml of PBS, 1 Nonidet P-40. The beads will then be eluted 200 µl of 1% SDS.

10 In another embodiment, the present invention relates to a method of inhibiting various types of cellular interactions including macrophage T cell interactions and lymphocyte and macrophage interactions with endothelial cells. The invention further relates to a method of inhibiting CD44-monocyte IL1 release. These methods also involve the administration of an effective amount of the CD44 protein or derivative or portion thereof to an animal in need of such treatment.

20 CD44 protein suitable for use in the present method can be isolated from synovial tissue (preferably, human synovial tissue) or the protein can be produced recombinantly. Synthetic peptides reflective of discrete regions of the CD44 molecule can be made by standard techniques.

30 One skilled in the art will appreciate that the amounts to be administered for any particular treatment protocol can readily be determined. The CD44 protein, peptide or derivative can be administered together with a pharmaceutically acceptable carrier.

35 In yet another embodiment, the present invention relates to a method of transporting a drug or cytotoxic agent to a site of inflammation in an animal comprising administering to the animal the CD44 protein, or peptide or derivative

thereof, linked, preferably covalently, to the drug or cytotoxic agent.

5 Examples of drugs to be targeted to organ-specific sites of inflammation are non-steroidal anti-inflammatory agents, forms of glucocorticosteroids, and cytotoxic agents such as cyclophosphamide. By either incorporating these agents in liposomes bearing CD44 molecules, or by physically linking CD44 molecules to these drugs,
10 one could achieve selective targeting or homing of the drug-CD44 complexes to sites of upregulated CD44 expression, that is sites of inflammation.

In a further embodiment, the present invention relates to a method of transporting a drug or
15 cytotoxic agent to a site of inflammation in an animal comprising administering to the animal CD44 protein, or peptide or derivative thereof, and a drug or cytotoxic agent wherein both are incorporated into a liposome.

20 The present invention is described in further detail by the following non-limiting Examples.

EXAMPLES

The following protocols and experimental details are referenced in the Examples that
25 follow:

Synovial Tissue and Synovial Fluid. Synovial tissue was obtained as discarded tissue from the Duke University Department of Pathology at the time of joint surgery. Synovial fluid was
30 obtained as discarded fluid from the Duke University Clinical Immunology Laboratory at the time of arthrocentesis.

Histopathologic Techniques. Synovial tissues were processed, cut, and studied in indirect

immunofluorescence (IF) assays as previously described (Hale et al (1989) Arth Rheum. 32:22-30). An inflammation score was generated for each synovium using light and IF microscopy based on the degree of T, B, and monocyte infiltration, vessel proliferation, fibroblast and synovial lining cell proliferation as described (Rooney et al (1988) Arth. Rheum. 31:956-963, McCachren et al (1990) J. Clin. Immunol. 10:19-27). The degree of reactivity of CD44 and anti-fibronectin antibodies was graded 1+ to 4+ with 1+ signifying reactivity with $\leq 25\%$ of synovial tissue reactive, 2+ $> 25\%$ and $\leq 50\%$ of synovial tissue area reactive, 3+ $> 50\%$ and $\leq 75\%$ of synovial tissue area reactive, and 4+ $> 75\%$ of synovial tissue area reactive.

Monoclonal Antibodies. The following monoclonal antibodies were used: CD44 (A1G3 and A3D8) (Haynes et al. (1983) J. Immunol. 131:1195-1200, Telen et al. (1983) J. Clin. Invest. 71; 1878-1886), anti-fibronectin (FN-15, Sigma, St. Louis, MO) 187.1 rat anti-mouse kappa chain (ATCC, Rockville, MD), 35.1 (CD2) (Martin et al. (1983) J. Immunol. 131:180-185), 9-1 (CD2) (Bernard et al. (1986) Hum. Immunol. 17; 388-405), and P3x63/Ag8 ascites fluid as a negative control.

Flow Cytometry. Flow cytometric analysis was performed on synovial fluid cells using a Becton-Dickinson (Mountain View, CA) FACS STAR PLUS flow cytometer in IF assays, as described (Hale et al. (1989) Arth Rheum. 32:22-30, Haynes et al (1981) New Engl. J. Med. 304:1319-1323).

Characterization of CD44 from Synovial Tissue. Synovial tissue was thawed, homogenized with a Dounce homogenizer in 0.6-1.0 ml extraction buffer

(10 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, 20 µg/ml soybean trypsin inhibitor, 1 mM iodoacetamide, and 1 mM PMSF), and centrifuged, (15000 rpm x 1 minute). The protein content of supernatants (tissue extracts) was determined using a copper/bicinchoninic acid assay (McCachren et al. (1990) J. Clin. Immunol. 10:19-27) (BCA Protein Assay, Pierce, Rockford, IL). Tissue extracts were analyzed by SDS-PAGE on 7% or 10% mini-gels (Mini-Protean II, Biorad Laboratories, Richmond, CA), followed by Western blot analysis using alkaline phosphatase-conjugated goat anti-mouse immunoglobulin along with the color development substrates BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitro blue tetrazolium) as developing reagents.

Western Blot Analysis of Tissue Extracts. To compare band densities from a given experiment, blots were photographed using TechPan film and resulting positive film densities measured using a laser densitometer. CD44 in trauma synovial tissue was given the value of 1 and the level of CD44 in RA and OA fluids were expressed as a ratio using the equation,

$$\text{CD44 ratio} = \frac{\text{CD44 in RA or OA Tissue}}{\text{CD44 in Trauma Tissue}}$$

Analysis of Synovial Fluid for CD44 Protein. Synovial fluid specimens were centrifuged, aliquoted, and stored at -80°C until processed. CD44 protein was immunoprecipitated from aliquots of synovial fluid which were precleared by incubation with P3-Sephadex (control) beads, then precipitated with either A3D8-Sephadex or P3-Sephadex. Immunoprecipitates were removed from the beads by boiling in 0.06 M Tris pH 6.8, 10% glycerol, 2%

SDS and analyzed by SDS-PAGE and Western blot analysis using alkaline phosphatase conjugated 187.1 rat anti-mouse immunoglobulin. The amount of CD44 in trauma synovial fluid was given the value of 1 and the level of CD44 in RA and OA fluids were expressed as a ratio using the equation,

$$\text{CD44 ratio} = \frac{\text{CD44 in RA or OA Fluid}}{\text{CD44 in Trauma Fluid}}$$

- 10 Band densities of CD44 in gels were determined as for tissue above.

Purification of Soluble CD44 Protein. CD44 (A3D8) and control YgG1 (P3X63/Ag8) antibodies were conjugated to CNBr-activated Sepharose CL-4B (Pharmacia, Piscataway, NJ) (3.0 mg IgG/ml gel). HuT 78 T Cell (CD44+) lysate was solubilized from 5×10^9 cells in 50 ml buffer (300 mM NaCl, 10 mM Na_2HPO_4 , pH 7.4, 0.2% NaN_3 w/v 0.5% NP-40 v/v), 0.01% Tween 80 w/v, 0.2 mM phenylmethylsulfonyl fluoride, and 0.1 mM tosyl L-lysine chloromethyl ketone) (0°C x 30 min), centrifuged (4°C 3000 x g x 15 min, 23420 x g x 30 min) filtered, precleared x 2 over a P3-Sepharose column, and allowed to bind overnight (4°C) to A3D8-Sepharose. The column was washed with 5 column volumes equilibration buffer, followed by 5 column volumes of 50 mM Tris, pH 7.4, 0.5% NP-40. NP-40 was exchanged for octyl glucoside (OG) (Sigma, St. Louis, MO) by washing with 2 column volumes 50 mM Tris pH 7.4, 1.5% OG w/v. CD44 protein was eluted with 2.5 M MgCl_2 , 50 mM Tris pH 7.4, 1.5% OG and the column regenerated by washing with 0.1 M Tris, 0.5 M NaCl, pH 8.5, then 0.1 M $\text{NaC}_2\text{H}_3\text{O}_2$, 0.5 M NaCl pH 4.5 and finally phosphate buffered saline (PBS). Eluted fractions were dialyzed sequentially against 50 mM Tris pH 7.4 + 1.5% OG, PBS + 1.5% OG, PBS + 1.25% OG, and PBS + 1% OG

4 using a ~~Centriprep-30~~ ^{CENTRIPREP} device (Amicon, Danvers, MA), and the affinity purification steps repeated until SDS-PAGE silver staining of the resulting CD44 protein preparation revealed only a single
5 band at 80-85 kD that reacted strongly with A3D8 antibody in Western blot analysis.

CD44 Liposomes. Liposomes were prepared by the method of Mimms et al ((1981) Biochemistry 20:833-840), using 1 μ M purified CD44 or control
10 glycoporphin protein, 1 nM L- α -dioleoyl lecthin (Avanti Polar Lipids, Birmingham, AL), and 240 nM OG. Liposomes were analyzed for content of the appropriate protein using a novel labelling technique and flow cytometry. Liposomes were
15 incubated with 5-(N-octadecanoyl) aminofluorescein (Molecular Probes, Eugene, OR) in PBS x 10 min at room temperature. Fluoresceinated liposomes were then reacted with 4.5 mm magnetic beads (Dynabeads M-450 Goat anti-Mouse IgG, Dynal Inc., Great Neck,
20 NY) coated with CD44 (A3D8) or anti-glycoporphin (E3,E4,E5) mAbs. After 45 minutes (4°C), with continuous end-over-end rotation, beads were washed x3 in PBS using a magnet to immobilize the beads during PBS changes. Fluoresceinated
25 liposome-bead conjugates were then analyzed by flow cytometry.

T Cell Activation Assay. PBMC from healthy donors were stimulated with optimal mitogenic concentration of CD2 mabs 35.1 and 9-1 as
30 described (Denning et al). Where indicated, CD44 or control glycoporphin liposomes (final protein range used was 28-140 nM) were added to these cultures 20 min prior to addition of CD2 mabs. In some experiments PBMC were pretreated with 0.1%
35 bromelain to remove cell membrane CD44 (Telen et

al (1983) J. Clin. Invest. 71:1878-1886, Hale
(1989) Immunol. 143:3944-3948).

EXAMPLE 1

Histologic Analysis of Expression of CD44 and Fibronectin in Trauma Synovium, OA and RA

CD44 expression was studied in synovial tissues from 7 RA and 8 OA patients who had surgery for joint replacement and 6 patients who had joint surgery due to joint trauma (Table 2). As a control for CD44, synovial tissue expression was also studied of the extracellular matrix protein, fibronectin. Previous studies have shown fibronectin deposition to be increased in synovial tissues in RA (Scott et al. (1991) Brit. J. Exp. Pathol. 62:362-368). Both CD44 and fibronectin expression were found to be dramatically unregulated in RA synovial tissue compared to their expression in OA or non-inflamed trauma synovium (Figure 1). In trauma and OA synovium, CD44 and anti-fibronectin mabs reacted with synovial lining cells, vessels and fibroblasts (Figures 1A-F). In RA, infiltrating lymphocytes and macrophages, as well as synovial lining cells, vessels and fibroblasts were brightly CD44+ (Figures 1G,H,I). In RA with pannus formation, both CD44 and fibronectin were widely expressed throughout synovial tissues (Figures 1K,J,L). RA tissues studied had a mean inflammation score of 13.1 ± 2.0 versus 6.6 ± 1.3 in OA tissues.. The degree of CD44 mAb reactivity in indirect IF assay was graded (CD44 index, see Methods) on a 1-4 scale with 1 the least CD44 present and 4 the most. The mean CD44 index in RA was $3.6 \pm .2$ versus $1.8 \pm .2$ in OA ($p < .001$) (Table 3). Thus, CD44 upregulation in synovial tissues in RA was due to two separate mechanisms: 1) increase in expression of CD44 on

synovial tissue cell types (synovial lining cells, vessels, fibroblasts), and 2) influx of CD44+ infiltrating immune cells (CD44+ T and B lymphocytes, macrophages).

Table 2
Characteristics of Patients From Whom Synovial Tissue Was Obtained For Study

Fibro. Patient Index	Age	Disease	Duration	Medications at Surgery	Tissue Site	Inflam. Score	CD44 Index
<u>RA</u>							
154 4+	68	RA	8 yrs	Pred	Knee	21	4+
146 4+	60	RA	>20 yrs	Pred, Mtx	Knee	13	4+
90 3+	61	RA	5 yrs	NSAID, Mtx	PIP	14	3+
86 4+	72	RA	>10 yrs	NSAID, ASA	Knee	18	3+
38 4+	68	RA	16 yrs	NSAID, ASA	Knee	8	4+
7 4+	62	RA	10 yrs	ASA, Pred,	PIP	13	4+
127 4+	59	RA	15 yrs	NSAID, Au Pred, TLI	Shoulder	5	3+
<u>OA</u>							
20 4+	58	OA	10 yrs	ASA, IAS	Knee	8	2+
11 3+	71	OA	10 yrs	ASA	Knee	8	2+
36 1+	60	OA	5 yrs	None	Hip	5	1+
198 2+	60	OA	2 yrs	NSAID	Hip	3	2+
242 4+	78	OA	8 yrs	NSAID	Knee	14	3+
244 2+	71	OA	13 yrs	NSAID	Knee	4	2+
169 2+	81	OA	7 mo.	NSAID	Shoulder	8	1+
237 1+	74	OA	20 yrs	NSAID	Knee	3	1+
<u>Trauma</u>							
148 1+	72	Trauma	4 mo.	NSAID	Shoulder	5	1+
212 4+	42	Trauma	1 yr	NSAID	Elbow	25	3+
211 4+	22	Trauma	6 mo.	None	MTP	10	3+
229 2+	35	Trauma	1 yr	ASA	Toe	5	1+
161 1+	36	Trauma	19 yrs	None	PIP	1	1+
250 1+	52	Trauma	9 mo.	None	MCP	4	1+

Pred, prednisone

Mtx, methotrexate

NSAID, non-steroidal anti-inflammatory agent

ASA, aspirin

Au, gold therapy

TLI, total lymphoid irradiation

IAS, intraarticular steroids

The diagnosis of RA was made using ACR criteria.

Mean Inflammation Score, CD44 Index and Fibronectin
Index in RA, OA, and Traumatic Synovium Tissues'

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Disease	Inflammation Score	CD44 Index	Fibronectin Index
Trauma (n=6)	9.2±4.2#	2.0±0.4	2.4±0.6
OA (n=8)	6.6±1.3	1.9±0.2	2.3±0.6
RA (n=7)	13.1±2.0	3.6±0.2"	3.8±0.1"

* all values mean ± SEM

** p<.001 when compared to trauma or to OA

15 # trauma mean inflammatory index varied from specimen to specimen, range (1-25). No. 229 (inflammation index = 5) was used as a control tissue in biochemical studies.

EXAMPLE 2

Direct Quantitative Assay of the Relative Amount of CD44 Protein in Synovial Tissue

The relative amount of CD44 in synovial
5 tissue was determined in 5 OA (nos. 244,169,237,242,
and 148), and in 3 RA (nos. 154,86, and 7) tissues
using quantitative Western blot analysis (Table 4).
Analysis of the 5 OA synovial tissues demonstrated a
mean relative amount of CD44 by Western blot of
10 3.5±0.7 (ie an average of 3.5x more CD44 than in
trauma synovial tissue no. 229). In contrast, RA
synovial tissues contained a mean relative amount of
CD44 by Western blot of 10.7±1.7 (Table 4). A
representative Western blot of CD44 levels in trauma
15 synovium (no. 229), RA synovium (nos. 7,154, and 86)
and in a representative OA synovium (no. 198) is
shown in Figure 2A. Figure 2B shows the relative
amounts of CD44 in each synovial tissue as determined
by the actual value obtained by laser densitometry of
20 the same Western blot gel. Thus, quantitative
Western blot analysis demonstrated RA tissue
contained 3 fold more CD44 per gram of wet tissue
than did OA tissue, 11 fold more than trauma
synovium, and demonstrated that the amount of
25 synovial tissue CD44 correlated with the degree of
inflammation present (Table 4).

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Table 4
Mean Relative CD44 Protein Levels in OA and RA
Synovial Tissues Determined by Western Blot Analysis

5	Disease	Inflammation		CD44 Protein Level#
		Score	CD44 Index'	
	OA (n=5)	6.4±2.1 [†]	2.0±0.3	3.5±0.7
	RA (n=3)	17.3±2.3	4.0±0.0 [@]	10.7±1.7 ^{**}

* CD44 index determined by histologic IF analysis.

10 # CD44 protein level determined by quantitative Western blot analysis. Data are arbitrary units relative to CD44 levels found in trauma synovium no. 229 (relative CD44 level =1, inflammatory index=5).

@ p<.01 when compared to OA.

15 ** p<.005 when compared to OA.

EXAMPLE 3

Comparison of the Relative Levels of Soluble CD44 in RA Versus OA Synovial Fluid

Because CD44 protein has been shown to circulate
5 in a soluble form in plasma and serum (Telen et al
(1983) J. Clin. Invest. 71:1878-1886), a
determination was made whether the soluble CD44 was
present in trauma, RA and OA synovial fluid. For
this analysis, immunoprecipitation of CD44 antigen
10 from 200 μ l of synovial fluid followed by
quantitative Western blot analysis was used. In 5 OA
synovial fluids studied, the mean WBC was 1250 ± 577
cells/mm³ and the mean level of CD44 was $.94 \pm 2$ [ie,
was $.94 \times$ the level of CD44 found in trauma synovial
15 fluid no. 11]. In RA synovial fluid, the mean cell
count was elevated ($11,279 \pm 2107$) ($p < .025$ compared to
OA) and the mean CD44 level was near double that of
trauma and OA synovial fluid ($1.91 \pm .4$) ($p < .001$)
(Table 5). Figure 3 shows examples of CD44 in RA
20 versus OA and trauma synovial fluids. Thus, RA
synovial fluid contained an average of 2 fold more
soluble CD44 than OA or trauma synovial fluid, and
the mean RA synovial cell count was higher than for
OA (Table 5). However, when individual RA synovial
25 fluid cell counts were plotted versus the relative
level of RA synovial fluid CD44, a significant trend
was observed to be present such that higher CD44
levels occurred in synovial fluid samples with lower
cell counts (Spearman Rank order correlation, $r = -.68$,
30 $p < .01$) (Figure 5). When RA synovial fluids were
grouped according to cell count, RA synovial fluids
with low cell counts (< 7000 cells/mm³) had $3.3 \times$
(2.84 ± 0.3 mean \pm SEM relative CD44 level) more CD44

than did RA synovial fluids with higher cell counts
(> 8500 cells/mm³) ($.85 \pm .35$ mean \pm SEM relative CD44
level). Thus, higher levels of soluble CD44 were
present in RA synovial fluids with lower cell counts,
5 and synovial fluid CD44 decreased to sub-normal
levels in the more inflammatory RA synovial fluids.

Table 5
Quantitation of CD44 Levels in OA and RA
Synovial Fluid

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	Patient	Diagnosis	Cell Count (mm ³)	CD44 Protein Level
5	18	RA	1,634	1.55
	31	RA	4,047	2.87
	25	RA	4,776	3.45
	6	RA	NA	4.81
10	26	RA	5,737	3.74
	22	RA	6,080	2.55
	30	RA	6,820	2.87
	15	RA	8,565	0.54
	23	RA	9,472	1.05
15	33	RA	9,772	0.70
	13	RA	11,061	1.69
	17	RA	19,840	0.00
	12	RA	20,214	0.47
	35	RA	23,867	1.31
20	34	RA	26,024	1.04
	mean \pm SEM		11,279 \pm 1,107	1.91 \pm 0.36
	5	OA	98	1.38
	16	OA	798	1.24
	24	OA	831	0.57
25	10	OA	1,072	0.73
	29	OA	3,469	0.77
	mean \pm SEM		1,250 \pm 577	0.94 \pm 0.16

* Data are arbitrary units relative to CD44 level found in trauma synovial fluid no. 11 (cell count 450, relative CD44 protein level taken as 1.0). The types of medications taken by RA patients with WBC >8500 did not differ from medications taken by RA patients with WBC <7000. RA Patient 17 also had calcium pyrophosphate crystals present in join fluid.

EXAMPLE 4

Effect of Elevated Synovial Fluid Cell Counts on Soluble CD44 Levels in Non-RA Forms of Inflammatory Synovitis

5 To determine if elevated synovial fluid cell
counts of $>8500/\text{mm}^3$ were associated with normal or
depressed synovial fluid CD44 levels in diseases
other than RA, 3 non-RA inflammatory synovial fluids
were studied, no.14 (Staphylococcus aureus septic
10 arthritis, cell count, $371,915/\text{mm}^3$), no. 32 (gout,
cell count, $35,400/\text{mm}^3$), and no.100 (psoriatic
arthritis, cell count, $9294/\text{mm}^3$). CD44 levels were
elevated in all three cases above the mean CD44 found
level in high-cell-count RA fluid (no. 14, CD44 level
15 = 2.45; no.32, CD44 level = 3.79; no. 100, CD44 level
= 7.00) (Figures 5A,5B). Thus, decrease of RA
synovial fluid CD44 levels in the presence of higher
($>8500/\text{mm}^3$) cell count was not a general phenomenon
related solely to the number of synovial inflammatory
20 cells present.

EXAMPLE 5

Effect of Soluble CD44 Protein on T Cell Activation

Because soluble CD44 antigen levels decreased in
RA synovial fluids with high cell counts but not in
25 gout or in other non-RA inflammatory synovial fluids,
and because cell-associated CD44 is involved in T
cell activation (Denning et al. ⁽¹⁹⁸⁹⁾ J. Immunol. ^{142:2988-97} 142:2988-97,
143:798-801, Shimuzu et al ^{143:798-801} (1989) J. Immunol.
143:2457-2463), potential immunologic sequelae of
30 decreased levels of soluble CD44 in inflammatory RA
synovial fluids on T cell function were determined.
To directly assess the effect of soluble CD44 protein
on T cell activation, CD44 was affinity-purified from
T cell membranes, incorporated into liposomes, and

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incubated with peripheral blood mononuclear cells
(PBMC) prior to stimulation with CD2 antibodies (a
potent stimulus of T cell activation) (Denning et
al., ^{(1989) J. Immunol. 142-2988-97} Stamenkovic et al. (1989) Cell 56:1057-1062).

5 Initial experiments showed that CD44
protein-containing liposomes alone had no effect on
the proliferation of T cells (data not shown).
However, when CD44 protein-containing liposomes (CD44
concentration 140nM) were added to PBMC prior to
10 addition of CD2 antibodies, T cell proliferation was
significantly decreased by $35 \pm 4\%$ ($n=4$, $p<0.02$) (Table
6) as compared to addition of control
(glycophorin-containing) liposomes. The cysteine
protease, bromelain, was previously shown to remove
15 surface CD44 from T cells (Telen et al., Hale et al
(1989) Immunol. 143:3944-3948). As a control, no
suppression of T cell activation was observed when
CD44 protein-containing liposomes were added to
bromelain-treated PBMC (average suppression $.5 \pm 7\%$,
20 $n=3$, $p=NS$) (Table 6).

Table 6

Effect of Soluble CD44 Protein
on CD2-Mediated T Cell Activation

	Treatment	Additions to Culture	cpm/10 ⁶ Cells
5	Sham treated PBMC	Media	460
		CD2 mAb	308,230
		CD2 mAb+CD44 liposomes	172,960
		CD2 mAb+control liposomes	255,090
10	Bromelain-treated PBMC	Media	5,380
		CD2 mAb	552,440
		CD2 mAb+CD44 liposomes	646,830
		CD2 mAb+control liposomes	550,000

* Data shown is from a single experiment representative of 4 experiments with sham-treated cells and 3 matched experiments with bromelain-treated cells. Protein concentration for both CD44 and glycophorin liposomes added in this experiment was 140 nM. The mean suppression of CD2 proliferation by sham-treated PBMC in the presence of CD44 liposomes in 4 separate experiments was $35 \pm 4\%$ (mean \pm SEM) as compared to control liposomes ($p < 0.02$, paired t-test). Addition of CD44 liposomes to bromelain treated PBMC had no significant effect on CD2-mediated proliferation (mean increase $5 \pm 7\%$, $p = \text{NS}$) as compared with control liposomes. Identical results were obtained when soluble, free CD44 protein was added to CD2 stimulated PBMC cultures (Data not shown).

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In a further embodiment, the present invention relates to a method of preventing or treating HIV infections.

5 HIV causes the acquired immunodeficiency syndrome (AIDS) and death in humans. Treatments to prevent and/or eliminate infection in humans are quite limited, with the only proven beneficial treatment being zidovudine. *In vitro* studies have documented that HIV infects human lymphocytes and mononuclear
10 phagocytes by way of adherence of virus gp 120 to cellular membrane CD4. CD4 or anti-CD4 antibodies block HIV infection, but this is usually not complete. Auxiliary cellular receptors for HIV (other than CD4) have been postulated, but these have
15 not been demonstrated. Clinical studies with soluble CD4 given to humans are being done now; preliminary results are apparently demonstrating no significant benefit.

This aspect of the present invention provides a
20 new method, based on a separate molecular pathway, of blocking HIV infection. The invention is based, at least in part, on Applicants' observation that CD44 (the hyaluronate receptor) facilitates HIV infection/expression in human cells. When this
25 molecule is "blocked" by binding to the anti-CD44 antibody A3D8 or AlG3, there is a 40-80% reduction of HIV infection/expression in normal human monocytes *in vitro*, as determined by inhibition of monocyte polykaryon formation and expression of viral reverse
30 transcriptase in supernatant medium. Likewise, hyaluronate (or hyaluronic acid), the natural ligand of CD44, inhibits infection/expression up to 85% (ID50=5 μ g/ml). Chondroitin sulfate, a polyanion which does not bind to CD44, has no or minimal
35 inhibitory activity.

Accordingly, this aspect of the present invention broadly relates to a method of preventing/treating HIV infection comprising contacting cells susceptible to HIV infection with an agent that inhibits HIV entry into such cells. Agents suitable for use in the above-described method include anti-CD44 antibodies (e.g., A3D8 or A1G3); soluble CD44, including recombinantly produced CD44; CD44 oligopeptides (e.g., CD44 peptides 1-10 set forth in Table 1); and hyaluronate. Chimeric molecules of CD44 with immunoglobulin can also be used, such molecules producing a lengthened circulation of CD44. These agents can be used alone or in combination with, for example, other compounds that block cellular receptors for HIV infection (e.g. soluble CD4) as well as reverse transcriptase inhibitors, such as zidovudine.

The agents suitable for use in the present method can be used alone or in combination, for example, for post-exposure prophylaxis therapy. Administration can be parenteral or loco-regional (for example, intravaginally).

One skilled in the art will appreciate that the above-referenced agents can be used in combination with alternative forms of HIV therapy to, for example, decrease the number of HIV-infected cells or to decrease the spread of virus from cell to cell. These agents can also be used to lessen the infectability of HIV in products designed for infusion into humans (e.g., blood derived transfusion products). Further, these agents can be used to prevent HIV infection or spread of HIV infection in cells, including human cells in vitro (e.g., blood cells). In addition, these agents can be used as

reagents for the experimental analysis of HIV infection of cells in vitro.

This aspect of the invention is described in further detail in the following non-limiting Example.

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EXAMPLE

To determine the importance of other monocyte cellular surface molecules in *in vitro* infection with HIV-1, the abilities of various murine monoclonal antibodies directed toward different monocyte membrane antigens to influence HIV-1 infection of human monocytes in vitro were determined. Monocytes from normal people were isolated by density gradients, adherence, and washing. Cells were inoculated with HIV-1_{8a.1} with or without three weeks of culture for reverse transcriptase (RT) activity, and the plates were examined morphologically. Antibodies A1G3 and A3D8 (anti-CD44 antibodies) reduced HIV-1-induced cytopathology and decreased supernatant RT, whereas an anti-Class I MHC antibody (3F10) had no effect. With A3D8, this effect appeared to be dose-related and disappeared gradually with increasing dilutions of the antibody (1:100 to 1:400). Monocytes treated with the CD44 ligand, hyaluronic acid (1-125 μ g/ml), had diminished morphological changes and RT expression (ID₅₀ \approx 5 μ g/ml). Chondroitin sulfate, a polyanion which does not bind to CD44, had no effect.

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All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

5 While the foregoing invention has been described
in some detail for purposes of clarity and
understanding, it will be appreciated by one skilled
in the art from a reading of this disclosure that
various changes in form and detail can be made
without departing from the true scope of the
10 invention and appended claims.

